Congestive Heart Failure in Copper-Deficient Mice

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Copper Deficiency (CuD) leads to hypertrophic cardiomyopathy in various experimental models. The morphological, electrophysiological, and molecular aspects of this hypertrophy have been under investigation for a long time. However the transition from compensated hypertrophy to decompensated heart failure has not been investigated in the study of CuD. We set out to investigate the contractile and hemodynamic parameters of the CuD mouse heart and to determine whether heart failure follows hypertrophy in the CuD heart. Dams of FVB mice were fed CuD or copper-adequate (CuA) diet starting from the third day post delivery and the weanling pups were fed the same diet for a total period of 5 weeks (pre- and postweanling). At week 4, the functional parameters of the heart were analyzed using a surgical technique for catheterizing the left ventricle. A significant decrease in left ventricle systolic pressure was observed with no significant change in heart rate, and more importantly contractility as measured by the maximal rate of left ventricular pressure rise (+dP/dt) and decline (–dP/dt) were significantly depressed in the CuD mice. However, left ventricle end diastolic pressure was elevated, and relaxation was impaired in the CuD animals; the duration of relaxation was prolonged. In addition to significant changes in the basal level of cardiac function, CuD hearts had a blunted response to the stimulation of the β-adrenergic agonist isoproterenol. Furthermore, morphological analysis revealed increased collagen accumulation in the CuD hearts along with lipid deposition. This study shows that CuD leads to systolic and diastolic dysfunction in association with histopathological changes, which are indices commonly used to diagnose congestive heart failure. Exp Biol Med 228:811–817, 2003

Key words: copper deficiency; congestive heart failure; heart contractility; isoproterenol; β-adrenergic response

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Cardiomyopathy associated with copper deficiency has been extensively investigated. The most notable early response to dietary copper restriction is the initiation and progression of heart hypertrophy (1–5). This hypertrophy is primarily of a concentric nature where the walls of the ventricles and the septum are enlarged with no change or a slight decrease in ventricular lumen size. This type of hypertrophy is mainly associated with pressure overload conditions such as hypertension and aortic stenosis (6–7). There are several stages in the development of a hypertrophic heart, which are followed by decompensation and eventually heart failure (1, 6). In the early stage, an increase in protein synthesis occurs, as well as an increase in the number and enlargement in the volume of mitochondria followed by an increase in myofibrils (1). In the second and stable stage of compensated hypertrophy, there is an increase in myofibrillar growth, and the ratio of mitochondria to myofibrillar volume densities remains normal (1). The third stage is decompensated hypertrophy and congestive heart failure where mitochondria-to-myofibrillar ratio is decreased with an imbalance between energy production and demand. During this stage of cardiac failure, myocardial stiffness and decreased contractility ensue because of fibrosis and increased interstitial collagen deposition (1). The hallmarks of heart failure are depression of contractility as measured by a decreased maximum rate of rise of left ventricular pressure (+dP/dt) during contraction and a significant increase in left ventricular end diastolic pressure (EDP) as well as various aberrant diastolic parameters (8–11). In addition the stage of cardiac failure is especially characterized by decreased β-adrenergic receptor density and a blunted response to adrenergic stimulation by catecholamines and various adrenergic agonists (12–14). Characterization of the electrophysiology of the copper deficient heart has been performed by several groups (3, 15, 16). Among the abnormalities in electrocardiographs of the copper deficient heart are aberrant ST segments, bundle branch block, supraventricular beats, ventricular beats, and wandering pacemaker (15). Increased P-R intervals and increased R wave duration were also reported (3, 16). An increase in H-V interval in His bundle electrography as well

as an increase in QRS amplitude and QT duration have also been reported (16). Although studies of the morphological (gross and ultrastructural) (30), electrophysiological and some molecular characteristics of the copper deficient heart are abundant, investigations have not produced satisfactory answers to an important question: would copper deficiency lead to congestive heart failure as demonstrated by depression in contractility and changes in hemodynamic parameters as well as a blunted response to β-adrenergic stimulation? In this report we use an *in vivo* technique to measure hemodynamic and contractile properties of the copper deficient mouse heart and investigate the response of the heart to adrenergic stimulation (17). We show for the first time that a transition does occur from compensated cardiac hypertrophy to decompensated heart failure where left ventricular EDP is increased, +dP/dt is decreased, diastolic function is altered and response to β-adrenergic stimulation is blunted.

Materials and Methods

Animals and Treatment. Friend Virus B-type (FVB) mice were bred and maintained at the University of Louisville animal facilities housed in plastic cages at 22°C on a 12-hr light/dark cycle. Dams of the pups were fed copper-deficient (CuD) AIN-93 diet containing 0.37 ppm Cu or copper-adequate (CuA) diet containing 5.76 ppm Cu starting on the third day postdelivery. The diet was prepared according to previously published report (18). The primary ingredients are cornstarch (53%), casein (20%), sucrose (10%), and soybean oil (7%) with essential vitamins and minerals. After the pups were weaned on the 21st day after birth, they were fed the same diet until they were sacrificed at weeks 4 and 5 of age. Animals had free access to double distilled water. Cages, feeding jars, and water bottles were rinsed regularly with water containing EDTA first and then with distilled water. Body weight was monitored weekly starting from the third week after birth. Only female mice 4 weeks of age were used for the functional analysis and female 4 and 5 weeks of age were used for other determinations. All procedures were approved by the AAALAC certified University of Louisville Institutional Animal Care and Use Committee.

Assessment of Left Ventricle Performance. Heart performance measurements were conducted using a surgical procedure previously described (17, 19). Mice were anesthetized by intraperitoneal injections of 60 mg of sodium pentobarbital/ kg BW. A midline incision (1–2 cm) in the throat area external to the trachea was made and the right and left sternohyoid muscles were pulled apart using forceps with serrated tips. A small opening in the trachea was made for the insertion of a PE-100 catheter to ensure a patent airway. The catheter was cut to a length of the animal's trachea to maintain normal resistance to airflow. The common right carotid artery, which is buried under the sternocleidomastoid muscle immediately adjacent to the sternohyoid muscles of the trachea, was isolated. The common artery was tied on one end at the branch point of the internal and external parts (towards the head) to prevent the backflow of blood from the peripheral vasculature of the head. The other end of the artery (towards the body) was clamped using a hemostat to occlude blood flow from the heart. A small incision is then made in the artery for the insertion of a hand-stretched PE-50 catheter. The catheter preparation and transducer connection are described below. The catheter was slowly advanced through the common carotid artery, the ascending aorta and into the left ventricle. When a waveform characteristic of ventricular pressure was achieved, the wound was covered to minimize liquid evaporation. The animal was then allowed to stabilize for 20-30 min before recording of waveform for up to 2 hr. At the end of each experiment, the chest was opened to confirm presence of catheter inside left ventricle.

The procedure for transducer connection is as follows: a PE-50 catheter to be inserted into the carotid artery was prepared by first soaking it in heparinized saline for 24 hr. One end of the catheter was cut on a bevel for easy insertion into the artery. The transducer was prepared by placing a three-way stopcock on each end. The stopcock and the transducer were filled with normal saline. A needle for insertion into the catheter was prepared by cutting off the tip to a length of 1.0 cm. The resulting needle was only 1.0 cm long. The needle hub was inserted into the blunt end of the catheter and it was connected to one of the three-way stopcocks.

Assessment of Isoproterenol Response. Isoproterenol was delivered through a femoral vein catheter (0.1 μl/g BW) with a microliter syringe pump (Harvard Aparatus-22). It was administered at a constant rate of infusion in varying concentrations of 0.08, 0.16, and 0.32 ng $Iso \cdot min^{-1} \cdot g\ BW^{-1}$ given for a total of 3 min for each dose. Animals were allowed to recover for 10–15 min before administration of each successive dose.

Assessment of Morphology Changes. At 4 and 5 wks of age female mice were anesthetized with Avertin (2,2,2 tribromoethanol) at 0.5 mg/g before being sacrificed. Hearts were excised, washed with saline solution and placed in 10% formalin. Hearts were cut transversely close to the apex to visualize the left and right ventricles. Several sections of heart (5 μm thick) were prepared and stained with hematoxylin and eosin for visualization using light microscopy. Heart sections were also stained with picro–Sirius red for the visualization of extracellular matrix collagen accumulation.

Cu Concentrations in the Heart and in the Diet. Cu concentrations were determined in the heart using inductively coupled argon plasma emission spectroscopy (model 35608, Thermo ARL-VG Elemental, Franklin, MA) after lyophilization and digestion of the tissues with nitric acid and hydrogen peroxide (32). Dietary Cu concentrations were analyzed by using a dry-ashing procedure, which was followed by dissolution of the residue in aqua regia and

measurement by atomic absorption spectrophotometry (model 503; Perkin–Elmer, Norwalk, CT).

Serum Ceruloplasmin. Serum ceruloplasmin concentrations were determined by its *p*-phenylenediamine oxidase activity (33). The oxidation of *p*-phenylenediamine at pH 5.4 yields a product that is readily detectable colorimetrically at 530 nm. The rate of the product formation is proportional to the concentration of ceruloplasmin.

Cu, Zn–Superoxide Dismutase (Cu, Zn–SOD). Total SOD activity was determined by a nitroblue tetrazolium assay (34). Briefly, tissue samples were homogenized in 19 vol of 0.5 M potassium phosphate buffer, pH 7.8. After centrifugation at 700*g* at 4°C for 10 min, the supernatant was collected and diluted 20-fold, and 50 μl of the diluted supernatant were applied to the assay. In each 1-ml assay cuvette, the following were added with the indicated final concentrations: potassium phosphate buffer (50 mM), pH 7.8; DETAPAC (1.0 mM); catalase (1.0 unit); nitroblue tetrazolium (5.6×10^{-5} M); and xanthine (0.1 mM). To initiate the reaction, xanthine oxidase (50 μl of 0.08 U/ml) was added. Absorbance at 560 nm was monitored for 6 min with a spectrophotometer (Beckman DU-650, Fullerton, CA). This procedure was also performed in the presence of NaCN (5 mM) to assay for Mn–SOD activity and the Cu, Zn–SOD was calculated by subtracting the Mn-SOD activity from the total SOD activity.

Data Analysis. A Student's *t* test was used for all experimental data analysis. Differences were considered significant at $P < 0.05$. The responses of the CuA and CuD groups to Iso stimulation were analyzed by comparing the regression coefficients using a Student's *t* test (35).

Results

To characterize the status of copper deficiency, copper levels in the heart, serum ceruloplasmin levels, Cu, Zn–SOD, and Mn–SOD activities in the heart were determined in 5-week-old CuA and CuD animals (Table I). Hearts of CuD animals had 3.5 times lower Cu levels and 4.6 times lower ceruloplasmin levels than CuA animals. The activity of the cuproenzyme Cu, Zn–SOD was markedly decreased in the CuD heart in comparison with the CuA (1.7 times lower in CuD). The activity of the non-copper containing Mn-SOD was not affected.

Cardiomyopathy associated with copper deficiency is characterized by concentric hypertrophy where ventricular walls and septum are thickened with no change in ventricular lumen size or a decrease in size. Table I shows the increase in absolute heart weight and in HW/BW ratio of CuD female mice at 4 and 5 weeks of age. The absolute heart weight and the HW/BW ratio of 4 weeks CuD mice were 1.4- and 1.5-fold higher than CuA mice, respectively. The values for 5-week-old CuD mice were 1.3- and 1.8-fold higher than CuA mice, respectively. Body weights were significantly different between the CuA and CuD groups at 5 weeks of age but not at 4 weeks. Observation of heart morphology by light microscope revealed the presence of vacuoles that possibly contain lipid droplets, a characteristic feature of cardiomyopathy of copper deficiency (Fig. 1A and B). The vacuoles were large enough to be visualized by light microscope, indicating a severe degree of damage in CuD hearts. In addition picrosirius staining of heart sections revealed greater collagen accumulation in the CuD heart compared to CuA controls (Fig. 1C and D).

Measurements of baseline functional parameters of the hearts of CuA and CuD mice indicate a compromised systolic function as shown by a significant decrease (17%) in the left ventricular peak systolic pressure (VPSP) and most importantly a significant and drastic decrease of 50% in +dP/dt in the CuD mice (Table II). In addition to systolic function, diastolic parameters were also changed with a significant increase of 315% in ventricular minimum diastolic pressure (VMDP) and 115% increase in end diastolic pressure (VEDP). The duration of relaxation was prolonged in CuD hearts relative to CuA controls (23% of CuA), and –dP/dt was decreased by 48%. In addition to a significant change in the duration of relaxation, a significant increase in Tau (53% of CuA) occurs in the CuD hearts. The parameter (Tau) is used for the determination of left ventricular stiff-

Table I. Comparison in Body Weight, Heart Weight, HW/BW ratio, Cu Concentrations, Serum Ceruloplasmin, Cu, Zn–SOD, and Mn–SOD Between CuA and CuD Female Mice

	Cu adequate		Cu deficient	
	Week 4	Week 5	Week 4	Week 5
Body weight (g)	17.1 ± 4	24.2 ± 3.5	16.4 ± 1.4	17.1 ± 0.7*
Heart weight (mg)	91.6 ± 9.5	126 ± 15	123.7 ± 28*	159 ± 22*Ψ
HW/BW (mg/g)	5 ± 0.8	5.2 ± 0.3	7.4 ± 1.8*	9.3 ± 1.3*Ψ
Cu concentration in heart (μg/g dry wt)	ND	25.1 ± 2.7	ND	7.1 ± 3.3*
Serum Ceruloplasmin (μg/ml)	ND	109.3 ± 14.3	ND	23.7 ± 9.6*
Cu, Zn–SOD (U/mg protein)	ND	150.9 ± 8.7	ND	88.5 ± 12.6*
Mn–SOD (U/mg protein)	ND	4.9 ± 1.6	ND	4.4 ± 1.1

Values are means ± SD (for CuA 4 week group $n = 5$; for CuA 5 week group $n = 6$; for CuD 4 week group $n = 11$; for CuD 5 week group $n = 4$ for first three parameters and $n = 6$ for the rest).
* Significantly different from CuA mice ($P < 0.05$).
Ψ Significantly different from CuD 4-week-old mice.
ND = not determined.

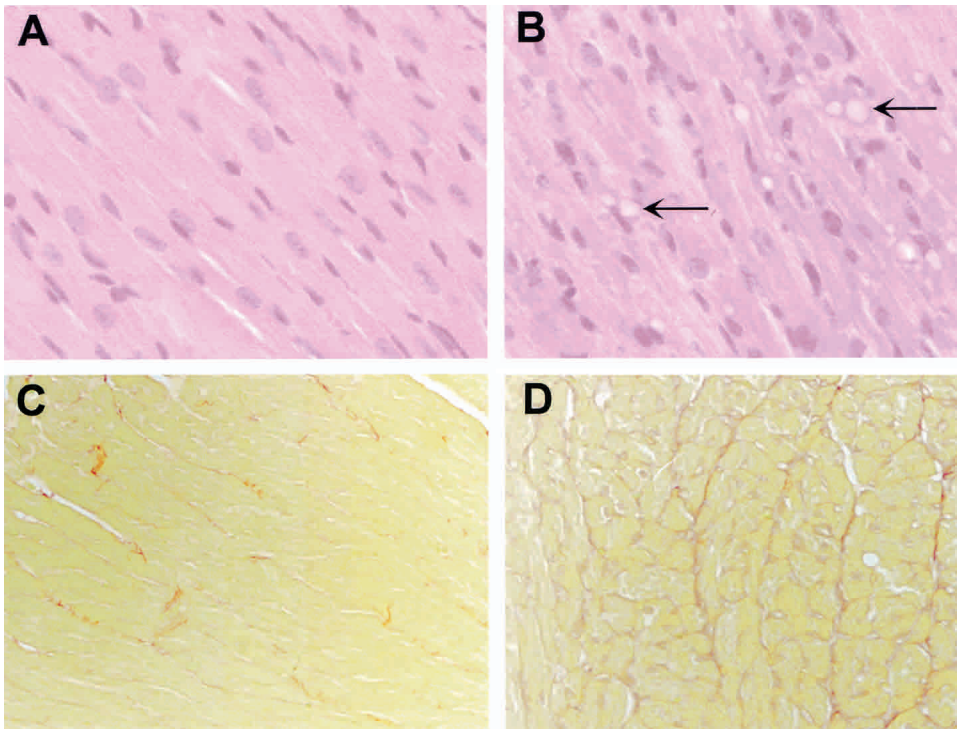

Figure 1. Histopathological examination of heart tissues of 5-week-old female mice. Tissue slides were stained with hematoxylin and Eosin (A and B) and Picro–Sirius Red (C and D). A-CuA heart, B-CuD heart (400x). Arrows point to vacuoles (possibly lipid droplets) that are large enough to be visualized using light microscopy. C-CuA heart, D-CuD heart (200x) stained with Picro-Sirius Red for visualization of collagen fibers.

Table II. Baseline Hemodynamic and Contractile Measurements of CuA and CuD Female Mice at 4 Week of Age

	CuA	CuD
HR, beats/min	477 ± 31	441.6 ± 37
VPSP, mm Hg	98.9 ± 5.0	81.8 ± 8*
VMDP, mm Hg	–1.44 ± 2.0	3.1 ± 1*
VEDP, mm Hg	3.3 ± 0.75	7.0 ± 0.8*
+dP/dt, mm Hg/s	7969.8 ± 858	4059 ± 889*
DCON, ms	34.8 ± 11	43.2 ± 6
Tau, ms	11.5 ± 1.7	17.6 ± 4*
1/2 R, msec	34.9 ± 7.0	34.5 ± 4
–dP/dt, mm Hg/s	6288.1 ± 879	3243.6 ± 737*
DREL, msec	56.1 ± 5.0	69 ± 5*

HR, heart rate; VPSP, ventricular peak systolic pressure; VMDP, ventricular minimum diastolic pressure; VEDP, ventricular end diastolic pressure; DCON, duration of contraction, ½ R, duration of ½ relaxation; DREL, duration of relaxation.
Data are expressed as mean ± SD.
CuA, $n = 6$; CuD, $n = 7$.
* Significantly different from CuA group ($P < 0.05$).

ness and represents the time constant of the best-fit exponential pressure decay from VPSP to a positive pressure above the previous VEDP by 10 mm Hg or a duration of 20 ms, whichever happens first. A slight decrease in HR was observed in the CuD hearts but the change was not statistically significant. No change in the duration of contraction was observed between the two groups.

It has been previously shown that β_1-adrenergic receptors are downregulated relative to β_2-receptors in cardiac failure (31). This reduces adrenergic responses elicited by adrenergic agents such as norepinephrine and isoproterenol (Iso) to the heart. In our study three different concentrations of Iso were used to assess the sensitivity of CuD hearts to adrenergic stimulation. As shown in Figure 2A–C, the response of CuD hearts to Iso stimulation was almost completely blunted whereas the CuA hearts showed gradual increases in HR, +dP/dt, and –dP/dt. Analysis of the regression coefficients from the linear regression equations calculated from the CuA and CuD data groups revealed a significant difference between the two groups ($P < 0.05$, Student's t test) (35). Other parameters such as VPSP, VEDP, and DREL also displayed a blunted response to Iso stimulation in the CuD hearts as shown in Table III.

Discussion

It is shown in this investigation that copper deficiency alone in an experimental rodent model leads to hypertrophic cardiomyopathy followed by congestive heart failure. Direct measurements of cardiac function revealed characteristics of heart failure with both diastolic and systolic dysfunction. In particular, cardiac contractility was severely depressed by copper deficiency. Hearts of CuD animals had a blunted response to isoproterenol, which suggests aberrant β-adrenergic signaling, a phenomenon frequently observed in end-stage cardiac failure patients as well as in experimental models of cardiac failure.

Heart hypertrophy is an adaptive response to stress and concentric hypertrophy is a specific response to pressure overload conditions (6–7, 24). The concentric nature of hypertrophy in CuD is unusual in that CuD is accompanied with valvular abnormalities or even dysfunction and anemia (1). One would expect an eccentric versus a concentric pattern of hypertrophy with these conditions because they lead

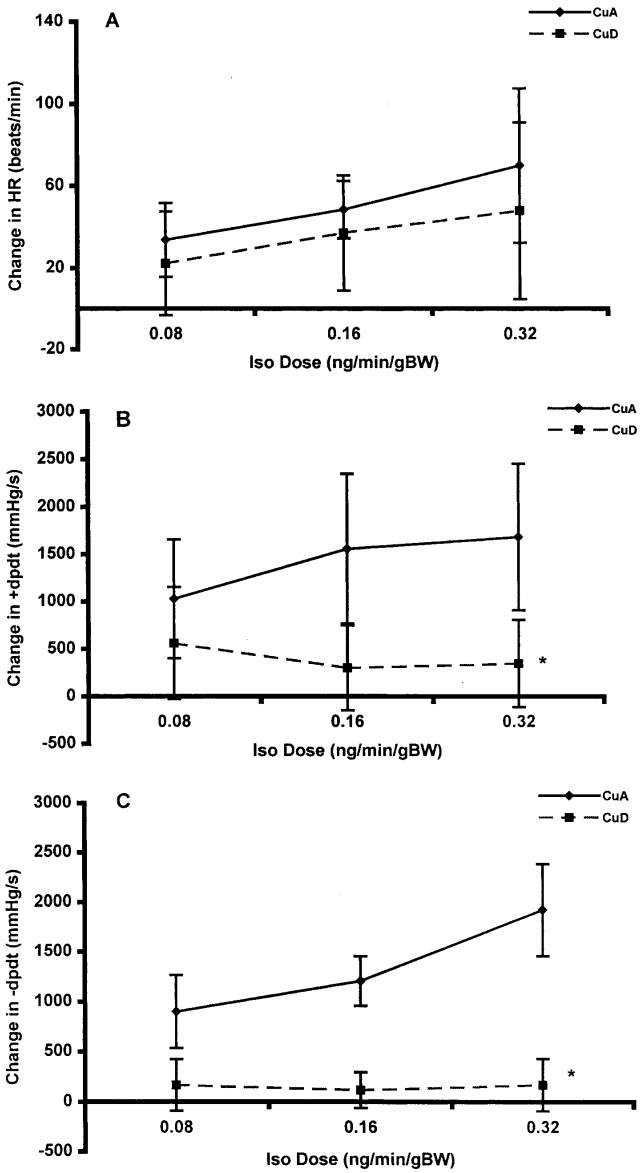

Figure 2. Response to Iso stimulation. A, change in HR; B, change in +dP/dt; C, change in –dP/dt. CuA (solid lines, $n = 6$); CuD (dashed lines, $n = 7$). * Significantly different from CuA control using Student's t test to compare regression coefficients from the linear regression equations ($P < 0.05$).

to a condition of volume overload. However, given the nature of hypertrophy in CuD, valvular dysfunction and anemia may not be the triggering factor in initiating the hypertrophic response in this pathological condition. It has been hypothesized that the concentric nature of hypertrophy of CuD may be related to abnormal connective tissue metabolism. This abnormality is associated with decreased activity of the cuproenzyme lysyl oxidase that leads to the reduction or inhibition of collagen and elastin crosslinking as well as an increased ratio of Type III to Type I collagen (27, 28). It is believed that the irregularity in connective tissue in CuD would lead to the following: 1) aortal distortion and loss of elasticity and ultimately rupture; 2) cardiac aneurysms; 3) hemothorax; 4) pleural effusion and rupture; or 5) hemopericardium (15). All of the aforementioned conditions are lethal and may be the cause of sudden death in CuD animals. However, at the late stage of congestive heart failure, there is increased collagen deposition and an increase in fibrosis, which lead to a decrease in elasticity and contractility of the heart muscle. The stage of compensated hypertrophy generally transitions to congestive heart failure in patients, which is characterized by both diastolic and systolic dysfunction as well as desensitization to β-adrenergic stimulation.

Diastolic dysfunction is characterized by slowed or incomplete relaxation, abnormal LV filling and altered passive elastic properties of the heart (25, 26). Myocardial relaxation depends mainly on the following factors: 1) inactivation processes within myocytes, i.e., the dissociation of actomyosin bridges and the lowering of Ca concentration by active uptake by the sarcoplasmic reticulum; 2) altered loading conditions, mainly afterload; and 3) restoring forces that depend on the elastic properties of the myocardium, which have been linked to extracellular matrix components such as collagen (29). The inactivation process that is responsible for Ca sequestration into the SR requires ATP and some studies have shown a decrease in ATP stores in the CuD heart (16), which may account for the prolonged relaxation time seen in our study. Several studies have shown that CuD results in aberrant collagen expression and this may lead to stiffness of left ventricle and therefore decreased elasticity and abnormal relaxation. Our study shows that copper deficiency results in an increase in cardiac stiffness as measured by the time constant of pressure decay, Tau. Another group using isolated rat cardiomyocytes has shown a decrease in the passive stiffness of individual cardiac myocytes as well as of papillary muscle tissue (36). Myocyte isolation involves the digestion of collagen and other extracellular fibrous components and therefore the stiffness referred to in the aforementioned study is that of intracellular cytoskeletal components. In our study staining for collagen has shown an increase in collagen accumulation in CuD hearts compared to CuA controls; this observation may provide an explanation for the increase in the time constant of pressure decay (Tau), i.e., stiffness of left ventricle, and the overall alteration in diastolic function.

Changes in MAX +dP/dt and VPSP are indicative of alterations in the systolic function of the left ventricle. The MAX +dP/dt, is an index frequently used to assess the mechanical ability of the heart to generate force for the ejection of blood from the ventricle. This parameter is increased with inotropic intervention such as treatment with digitalis glycosides or isoproterenol as well as an increase in heart rate that reflects the augmented level of contractility associated with tachycardia. Furthermore, an increase in preload (elevated end-diastolic volume and pressure) and afterload (increased aortic diastolic pressure) increase MAX +dP/dt under normal conditions. The decrease in MAX +dP/dt observed in CuD mice could indicate a decrease in contractility of left ventricle, which could be attributed to several

Table III. Cardiac Systolic and Diastolic Response to Isoproterenol Stimulation in CuD and CuA Female Mice at 4 Weeks of Age

Iso dose (ng/min/gBW)	0.08	0.16	0.32	*P*
Change in VPSP (mm Hg)				
CuA	+5.2 ± 3.0	+9.3 ± 3.0	+6.0 ± 2.3	<0.05
CuD	+1.3 ± 1.6	+0.75 ± 1.2	+1 ± 1.4	
Change in VEDP (mm Hg)				
CuA	–1.4 ± 0.4	–2.0 ± 0.5	–2.3 ± 1.2	NS
CuD	–0.3 ± 0.7	–1.5 ± 1.8	–1.5 ± 1.8	
Change in DREL (msec)				
CuA	–11.3 ± 7.0	–15 ± 9.0	–10.5 ± 7.3	NS
CuD	–2.5 ± 4.0	–4.3 ± 4.0	–5.2 ± 6.0	

CuA and CuD animals received three different doses of Iso and the response is expressed as either an increase or a decrease in the specified parameter.
VPSP, ventricular peak systolic pressure; VEDP, ventricular end diastolic pressure; DREL, duration of relaxation.
A Student's *t* test was used to compare regression coefficients from the linear regression equations.
CuA, $n = 6$; CuD, $n = 7$.

factors that have been previously observed in CuD heart rat: 1) aberrant mitochondria to myofibrillar ratio, which decreases supply of energy while demand is still high (ischemic injury) and 2) changes in the morphology of the heart with an increase in collagen deposits and distorted sparse myofibrils with poorly aligned Z bands (reduction of the basic contractile elements in the myocardium).

β-adrenergic receptor stimulation by catecholamines (norepinephrine) or adrenergic agonists (isoproterenol) leads to alterations in heart rate, force of contraction, rate of cardiac relaxation, and automaticity. β-adrenergic receptor belongs to the family of G-protein-coupled receptors that are regulated in response to external stimulation by expression and functional modulation. It has been suggested that alterations in β-adrenergic receptor signaling occur in congestive heart failure caused by cardiomyopathy. Alterations in the expression (degradation or downregulation of mRNA) and function (through phosphorylation) of the receptor, G protein, or adenylyl cyclase are possible mechanisms for blunting of the receptor response to agonists. The desensitization or the deactivation of the β-adrenergic receptor is thought to occur in heart failure because of continuous stimulation by an increased circulating amount of catecholamines. The increase in circulating neurotransmitters is caused by the activation of the sympathetic nervous system, which occurs in the development of heart failure (13, 20–24). In the current model of hypertrophic cardiomyopathy followed by congestive heart failure by CuD, the hearts displayed a blunted response to isoproterenol, which suggests that β-adrenergic receptor desensitization may also occur in CuD cardiomyopathy as it leads to congestive heart failure. Although previous studies have shown that copper deficiency results in the depletion of left ventricular norepinephrine, reasons for this could be related to species difference or the time point of measurement of norepinephrine (8-week-old rats) at which time the hearts could be well into the failure stage (37).

In summary, our investigation of cardiomyopathy associated with dietary copper deficiency has produced evidence of the transition from hypertrophy to cardiac failure. The physiological aspects of heart failure especially the desensitization of the β-adrenergic signaling pathway observed in this study, together with those of cellular events (previously investigated in our laboratory), such as the activation of the apoptotic machinery (5), demonstrate the uniqueness of this model for the study of concentric hypertrophy as well as the molecular and physiological aspects of the important transition from hypertrophy to cardiac failure.

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